Remarks

The Office Action mailed March 27, 2003 has been received and reviewed. Claims 21, 26, 31, 32, 34, 35, 38, and 41 having been amended, claims 18-20 and 28-30 having been cancelled, and claims 44 and 45 having been added, the pending claims are claims 21-27 and 31-45. Reconsideration and withdrawal of the rejections are respectfully requested.

Support for the amended claims is found throughout the specification. For example, support for the recitation "gastrointestinal alimentary tract" in amended claims 21, 31, 32, 34, 35, 38 and 41 is found at p. 5, line 12 of the specification; support for the recitation "suffering from a gastrointestinal disorder" in amended claims 21, 34, and 35 is found at p. 2, line 5 of the specification; and support for the recitation "enteric nervous disorder" in amended claims 38 and 41 is found at page 2, line 3 of the specification.

Page 2, line 14, of the specification has been amended to replace the abbreviation "SP" with the recitation "substance P," the complete terminology for the abbreviation SP. No new matter has been added with this amendment to the specification.

Examiner Interview

A telephonic interview was held June 27, 2003 between Examiner Joseph Woitach and Applicants' Representative Nancy Johnson. In this interview the outstanding Election of Species Requirement and the rejections under 35 U.S.C. §112, first paragraph, and 35 U.S.C. §102 were discussed. The Examiner is thanked for the courtesy of this interview.

Election/Restriction

Applicants continue to traverse the Election of Species Requirement, mailed May 1, 2002. Further, Applicants request clarification as to the scope of the claims currently under examination.

The Examiner issued a Restriction Requirement under 35 U.S.C. 121 on May 1, 2002, grouping the claims as follows: Group I, claims 1-16, drawn to a method of treating a

disorder comprising implanting stem cells, progeny thereof or combinations thereof, into the gastrointestinal organ of a subject; and Group II, claims 17-20, drawn to a method of producing insulin in a subject comprising implanting stem cells, progeny thereof or combinations thereof, into the gastrointestinal organ of a subject. In the Response to this Restriction Requirement, mailed May 22, 2002, Applicants elected Group I, claims 1-16, with traverse. In the Office Action mailed August 13, 2002, upon review and reconsideration, the Examiner withdrew the restriction requirement between Groups I and II and all original claims, claims 1-20, were examined on the merits. Applicants note with appreciation the withdrawal of this Restriction Requirement. Applicants submit that original claims 1-20 were all drawn to methods comprising the overall method step of implanting isolated stem cells, progeny thereof, or combinations thereof into a gastrointestinal organ of a subject. Further, Applicants submit that all currently pending claims are drawn to methods comprising the same overall method step of implanting stem cells, progeny thereof, or combinations thereof into the gastrointestinal alimentary tract of a subject. Thus, Applicants respectfully submit that all pending method claims are in the same Group as the claims as originally presented and searched and examined by the Examiner (see MPEP 818.02(a)).

The Restriction Requirement mailed May 1, 2002, also included an Election of Species Requirement. According to the Examiner, Group I was drawn to several specific disorders and Applicants were required under 35 U.S.C. 121 to elect a single disclosed species from the group of: (1) a degenerative disorder; (2) an immunological/inflammatory disorder; (3) a neoplastic disorder; or (4) an idiopathic condition, as set forth in original claim 16. In the Response to this Restriction Requirement, mailed May 22, 2002, Applicants elected species (1), a degenerative disorder, with traverse.

Applicants submit that pending claims 21-45 are generic claims and contain no recitations directed at the elected species (a degenerative disorder) or any non-elected species. Thus, Applicants request clarification of the following statements by the Examiner in the most recent Office Action:

(1) "Newly added claims 21-34 are drawn to a method of repopulating cells . . . and are broader than the original claims for treating a degenerative disorder" (page 2 of the Office Action mailed March 27, 2003);

(2) "[C]laims 21-34 . . . will be examined to the extent they encompass *the elected invention of a method for the treatment of a degenerative disorder*" (page 3 of the Office Action mailed March 27, 2003 (emphasis added)). Applicants note that this statement is incorrect, a degenerative disorder is the elected *species*, not the elected *invention*;

(3) "[C]laims 35-40 are drawn to a method of providing a neurotransmitter . . . and claims 41-43 are drawn to method of treating a disorder of the enteric nervous system, and though no specific degenerative disorder is set forth . . . they will be examined to the extent they encompass *the elected invention* of a method for the treatment of a degenerative disorder" (page 3 of the Office Action mailed March 27, 2003 (emphasis added)). Applicants again note that this statement is incorrect, a degenerative disorder is the elected *species*, not the elected *invention*;

(4) "[C]laims 41-43 will be examined to the extent that implanting cells into the intestine will treat an degenerative disorder, *in particular where the cells provide nitric oxide* (claim 42)" (page 3 of the Office Action mailed March 27, 2003 (emphasis added)). With this statement is the Examiner implying that the examination of claims 41-43 has been limited to the scope of language recited in dependent claim 42, language that has never been the subject of a restriction/election requirement; and

(5) "Claims 18-43 are pending and currently under examination as they are drawn to the elected invention of a method for treating a degenerative disorder" (page 4 of the Office Action mailed March 27, 2003). Applicants again note that this statement is incorrect, a degenerative disorder is the elected *species*, not the elected *invention*.

Applicants respectfully request clarification of the election of species requirement and of the scope of claims as examined by the Examiner. Applicants further note that the Examiner's statements as to the scope of the claims under examination are not supported by the

rejection of the claims under 35 U.S.C. §102(b), as anticipated by Luo et al. (claims 21, 22, 28, 29, and 32) or Keller et al. (claims 21, 22, 25, 26, 30, 31, and 32). Both Luo et al. and Kellor et al. are drawn to the treatment of healthy host animals by the transplantation of intact organs. Neither Luo et al. nor Keller et al. teach the treatment of the elected species, a degenerative disorder, or the treatment of any type of disorder what-so-ever. Thus, the Examiner's search and examination of the claims has not been limited to the elected species, a degenerative disorder. The Examiner has searched and examined the pending claims generically, as a method comprising the step of implanting stem cells.

Applicants respectfully submit that pending claims 21-27 and 31-45 are in the same restriction group (drawn to methods comprising implanting stem cells, progeny thereof, or combinations thereof into the gastrointestinal alimentary tract of a subject) as originally presented claims 1-20, and they have been searched and examined by the Examiner in the Office Action mailed August 13, 2002.

Claim Objection

The Examiner objected to claims 21-26 and 28-43 because of the following informalities: "[t]he election of species was made to a degenerative disorder, however the claims broadly encompass any disorder. The claims should be amended to reflect the elected invention" (page 5 of Office action mailed March 27, 2003). This objection is respectfully traversed. Applicants respectfully note that, according to MPEP § 809.02(a), Applicants' claims will be limited to a single, elected species only if no generic claim is finally held allowable. Thus, it is inappropriate, at this time, to require amendment of the claims, including generic claims, to limit the claims to the elected species only. Withdrawal of this objection of the claims is respectfully requested.

The 35 U.S.C. §112, First Paragraph, New Matter Rejection

The Examiner rejected claim 37 under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. This rejection is respectfully traversed.

The Examiner asserted that the recitation "substance P" in amended claim 37 introduces new matter. Specifically, the Examiner asserted that "literal support for the term 'substance P' is found in the specification at page 11, lines 10-11, however, this portion of the specification describes antibodies . . . for staining cells. The specification does not support providing the specific neurotransmitter 'substance P' by transplanting cells for any reason or in any context of treatment" (page 6 of Office Action mailed March 27, 2003).

Applicants respectfully disagree. The specification states on page 3, lines 13-20, "[s]pecifically, the present invention provides a method of implanting (e.g., transplantation) of stem cells . . . into a gastrointestinal organ . . . for the purposes of repopulating various cellular components . . . and/or providing a source of biological materials (e.g., neurotransmitters . . .) for therapeutic intent." Page 2, line 14, of the specification lists substance P (referred to by the abbreviation 'SP') as one of the neurotransmitters of the gastrointestinal tract. And, page 10, line 29, through page 11, line 11, of the specification states "[s]urvival of the graft in the living host can be examined using various methods . . . . Cells can be stained with any stains visible under light or electron microscopic conditions . . . . Most preferable are antibodies that identify any neurotransmitters, particularly those directed to GABA, TH, ChAT, and substance P." Thus, the specification provides clear support for methods of transplanting cells for the therapeutic intent of providing neurotransmitters, including the neurotransmitter substance P. Reconsideration and withdrawal of this rejection under 35 U.S.C. §112, first paragraph, is respectfully requested.

The 35 U.S.C. §112, First Paragraph, Enablement Rejection

The Examiner rejected claims 18-20 and 21-43 under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. This rejection is respectfully traversed.

Specifically, the Examiner asserted that the specification "provides no means to avoid the host's immune system which is a major source for graft rejection in xenotransplantation. Further, the evidence provided in the declaration does not address the problems of xenotransplantation" (page 8 of Office Action mailed March 27, 2003). Applicants respectfully disagree.

First, Applicants submit that the present specification exemplifies the successful xenogeneic transplantation of rat neural stem cells into the gastrointestinal wall of mice. See the Examples, page 13, lines 12-30, page 14, lines 8-12 and Figure 4.

Second, Applicants submit that the methods of the claimed invention are not limited to xenogenic transplantation. The specification teaches that stem cells can be obtained from the tissues of "a wide variety of animals, such as insects, fish, reptiles, birds, amphibians, mammals, and the like. The preferred source is mammals, preferably rodents and primates, and most preferably mice and humans" (p. 8, lines 4-7 of the specification).

Third, unfortunately, the purpose of evidence presented in paragraphs 5-8 and Exhibits A and B of the Declaration under 37 C.F.R. § 1.132 of Pankaj Jay Pasricha and Maria-Adelaide Micci (a copy of which was submitted with Applicants' previous response mailed January 3, 2003) has not been fully appreciated. While paragraph 5 of this Declaration begins with the statement "[t]o overcome the issues related to xenotransplantation," the information of paragraphs 5-8 and Exhibits A and B of the Declaration under 37 C.F.R. § 1.132 of Pankaj Jay Pasricha and Maria-Adelaide Micci was not submitted as an additional working example of xenogenic transplantation. Rather, this information of the successful isolation and implantation

of murine neural stem cells into a mouse, was presented as evidence of the successful application of the claimed method with *non-xenogenic* transplantation.

Finally, the specification (see p. 10, lines 3-18) provides clear teachings of the various methods of immunosuppression that can be used to reduce or eliminate the immune response that might occur with the implantation of stem cells into a subject. Such methods are well known to the skilled artisan and include the use of immunosuppressive drugs, such as cyclosporine, or the use of locally applied immunosuppressants. As an alternative to immunosuppression techniques, gene replacement or gene knockout methods can be applied to stem cells, to eliminate the need to immunosuppress the recipient. Applicants direct the Examiner to Low et al., "Immunobiology of Neural Xenotransplantation," in *Neural Transplantation Methods* Edited by Dunnett, Boulton and Baker; Humana Press 2000:503-541 (a copy of which is provided as Exhibit A) for a more complete discussion of immunosuppressive agents and therapies that are particularly applicable to neural xenotransplantation. Applicants further submit that Luo et al. (Xenotransplantation 1998 Aug;5(3): 197-206), cited by the Examiner in the rejection of claims 21, 22, 28, 29, and 32 under 35 U.S.C. §102 teaches successful liver allotransplantation and xenotransplantation.

Thus, Applicants submit that the specification provides adequate teachings of various methods of immunosuppression. Applicants further submit that such methods are well known to the skilled artisan. Reconsideration and withdrawal of this rejection of the claims under 35 U.S.C. §112, first paragraph, is respectfully requested.

On page 10 of the Office Action mailed March 27, 2003, the Examiner asserted that claims 21-41 "are only considered to the extent that they encompass the elected *invention* of treating a degenerative disorder (emphasis added). As presented in the "Election/Restriction" section above, Applicants respectfully submit that this is incorrect. A degenerative disorder is the elected *species*, not the elected *invention*, and the Examiner is improperly limiting the scope of generic claims to the scope of an elected species.

The Examiner also asserted that the specification provides "no guidance for providing cells to a normal subject who maintain a normal number of cells" (page 10 of Office Action mailed March 27, 2003. Applicants respectfully disagree. However, Applicants respectfully submit that this assertion is moot in view of the amendment of independent claims 21, 35, 38, and 41. Specifically, as amended, claim 21 is drawn to "[a] method of repopulating neurons, muscles, or other tissues within the gastrointestinal alimentary tract of a subject suffering from a gastrointestinal disorder comprising implanting isolated stem cells . . . into the gastrointestinal alimentary tract of the subject suffering from a gastrointestinal disorder"; claim 35 is drawn to "[a] method of providing a neurotransmitter within the gastrointestinal alimentary tract of a subject suffering from a gastrointestinal disorder comprising implanting isolated neural stem cells . . . into the gastrointestinal alimentary tract of the subject suffering from a gastrointestinal disorder"; claim 38 is drawn to "[a] method of producing nitrinergic neurons in a subject suffering from an enteric nervous disorder comprising implanting isolated neural stem cells into the smooth muscle of the gastrointestinal alimentary tract of the subject suffering from an enteric nervous disorder"; a claim 41 is drawn to "[a] method of treating a disorder of the enteric nervous system in a subject suffering from an enteric nervous disorder comprising implanting isolated neural stem cells . . . into the gastrointestinal alimentary tract of the subject suffering from an enteric nervous disorder."

Additionally, the Examiner asserted that "there is not indication that providing a neuronal cell will provide any form of treatment to a subject" (page 11 of the Office Action mailed March 27, 2003.) Applicants disagree. Exhibit D of the Declaration under 37 C.F.R. § 1.132 of Pankaj Jay Pasricha and Maria-Adelaide Micci provides evidence of the successful treatment of a subject by the methods of the claimed invention. Specifically, Exhibit D demonstrates that the implantation of neural stem cells into the pyloric wall of nNOS -/- mice (mice that serve as a model system of a gastrointestinal disorder and an enteric nervous system disorder) demonstrates a correction of the abnormalities in gastric physiology (shown by an improvement in the gastric emptying of both solids and liquids) present in this mouse model

system of a gastrointestinal disorder/enteric nervous system disorder. Thus, the implantation of neural stem cells is effective in correcting a neurotransmitter deficiency and producing a beneficial functional effect.

Further, the Examiner asserted that"[t]he specification is silent with respect to specific methodology for cell transplantation and provides no guidance for specific treatment of specific disorders" (page 12 of the Office Action mailed March 27, 2003). Applicants respectfully disagree. The methods of claims 21-27 and 31–43 are drawn to "implanting" neural stem cells into the gastrointestinal tract of a subject. Applicants submit that methods for such implantation into the gastrointestinal tract are well known to the skilled artisan.

On view of the above discussions, Applicants submit that the specification provides adequate teaching and guidance for the claimed methods. Reconsideration and withdrawal of the rejection of the claims 21-27 and 31-43 under 35 U.S.C. §112, first paragraph, is respectfully requested.

The 35 U.S.C. §112, Second Paragraph, Rejection

The Examiner rejected claims 18-20 and 32 under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. This rejection is respectfully traversed.

Applicants respectfully submit that the rejection of claims 18-20 is moot in view of the cancellation of claims 18-20. The Examiner included claim 32 in this rejection under 35 U.S.C. §112, second paragraph. Specifically, the Examiner asserted that "the metes and bounds of the term 'administering the cells locally' is not clearly defined (see page 15 of Office Action mailed March 27, 2003 (emphasis in original)). Applicants respectfully disagree. Claim 32 is drawn to a method "wherein implanting cells into a gastrointestinal alimentary tract of a subject comprises administering the cells locally." Applicant's respectfully submit that page 10, lines 19-22, of the specification, with the statement "[c]ells can be delivered throughout the affected area, in particular in a site-specific manner (i.e., locally) . . . . [c]ells can be administered to the

particular region using any method that maintains the integrity of the surrounding areas, preferably by local injection," clearly defines the recitation 'administering cells locally.' Applicant's respectfully submit that metes and bounds of the recitation 'administering cells locally' is clear. For the reasons discussed above, reconsideration and withdrawal of this rejection under 35 U.S.C. §112, second paragraph, is respectfully requested.

The 35 U.S.C. §102 Rejection

The Examiner rejected claims 21, 22, 28, 29, and 32 under 35 U.S.C. §102 as being anticipated by Luo et al. (Xenotransplantation 1998 Aug;5(3): 197-206). This rejection is respectfully traversed. Specifically, the Examiner asserted that Luo et al. teach a method of liver transplantation that is the same as the method of claims 21, 22, 28, 29, and 32. Applicants respectfully disagree. Claims 21, 22, 28, 29, 32 are drawn to a method "comprising implanting isolated stem cells, progeny thereof, or combinations thereof into the gastrointestinal alimentary tract of the subject suffering from a gastrointestinal disorder." Luo et al. teach the transplantation of an intact liver into the abdomen of a healthy subject. Luo et al. do not teach implanting "isolated stem cells, progeny thereof, or combinations thereof." Luo et al. do not teach implanting such isolated cells "into the gastrointestinal alimentary tract." Luo et al. do not teach implanting into a "subject suffering from a gastrointestinal disorder." Thus, the disclosure of Luo et al. does not set forth each and every element of claims 21, 22, 28, 29, and 32. Applicants respectfully submit that Luo et al. do not anticipate the method of claims 21, 22, 28, 29, and 32. Withdrawal of this rejection under 35 U.S.C. §102 is respectfully requested.

The Examiner rejected claims 21, 22, 25, 26, 30, 31, 32 under 35 U.S.C. §102 as being anticipated by Keller et al. (J Invest Surg 1997 Nov-Dec;10(6): 375-8). This rejection is respectfully traversed. Specifically, the Examiner asserted that Keller et al. teach a method of intestinc transplantation that is the same as the method of claims 21, 22, 25, 26, and 30-32. Applicants respectfully disagree. Claims 21, 22, 25, 26, and 20-32 are drawn to a method "comprising implanting isolated stem cells, progeny thereof, or combinations thereof into the

gastrointestinal alimentary tract of the subject suffering from a gastrointestinal disorder." Keller et al. teach the transplantation of an intact section of the small intestine into a healthy subject. Keller et al. do not teach implanting "isolated stem cells, progeny thereof, or combinations thereof." Keller et al. do not teach implanting such isolated cells "into the gastrointestinal alimentary tract." Keller et al. do not teach implanting into a "subject suffering from a gastrointestinal disorder." Thus, the disclosure of Keller et al. does not set forth each and every element of claims 21, 22, 25, 26, and 30-32. Applicants respectfully submit that Keller et al. do not anticipate the method of claims 21, 22, 25, 26, and 30-32. Withdrawal of this rejection under 35 U.S.C. §102 is respectfully requested.

Summary

It is respectfully submitted that the pending claims 21-27 and 31-45 are in condition for allowance and notification to that effect is respectfully requested. The Examiner is invited to contact Applicants' Representatives, at the below-listed telephone number, if it is believed that prosecution of this application may be assisted thereby.

Respectfully submitted for
Pasricha et al.

By
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July 25, 2003
Date

By: Nancy A. Johnson
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CERTIFICATE UNDER 37 CFR §1.8:
The undersigned hereby certifies that this paper is being transmitted by facsimile in accordance with 37 CFR §1.6(d) to the Patent and Trademark Office, addressed to Assistant Commissioner for Patents, Mail Stop AF, P.O. Box 1450, Alexandria, VA 22313-1450, on this 25th day of July, 2003, at 2:30pm (Central Time).

By: Jill R. Aguilar
Name: Jill R. Aguilar

APPENDIX A - SPECIFICATION/CLAIM AMENDMENTS INCLUDING NOTATIONS TO INDICATE CHANGES MADE
Serial No.: 09/834,110
Docket No.: 265.00090101

Amendments to the following are indicated by underlining what has been added and bracketing what has been deleted.

In the Specification

The paragraph beginning at page 2, line 8, has been amended as follows:

The enteric nervous system (ENS) is a part of the peripheral nervous system and consists of neuronal cell bodies, their fibers, and supporting cells located within the wall of the GI tract. These cell bodies are arranged in two major ganglionated plexuses, a peripheral myenteric (Auerbach's) between the circular and the longitudinal muscle layers, and a submucosal (Meissner's) plexus in the submucosal connective tissue between the muscularis mucosa and circular muscle. For the most part, the myenteric neurons provide excitatory (acetylcholine and [SP] substance P) and inhibitory (nitric oxide, VIP, CGRP, and ATP) transmitters to the gut smooth muscle. The tone of the gut muscle depends on the summated influence of the opposing actions of these neurotransmitters.

In the Claims

For convenience, all pending claims are shown below.

18. (CANCEL) The method of claim 19 wherein the stem cells are selected from the group of multipotent, pluripotent, totipotent stem cells, and combinations thereof.

19. (CANCEL) A method of producing enhanced levels of insulin in a subject comprising implanting neural stem cells and/or progeny thereof into the pancreas of the subject.

20. (CANCEL) The method of claim 19 wherein the stem cells and/or progeny thereof are derived from embryonic neural tissue, adult neural tissue, or combinations thereof.

21. (AMENDED) A method of repopulating neurons, muscles, or other tissues within [a] the gastrointestinal [organ] alimentary tract of a subject suffering from a gastrointestinal disorder comprising implanting isolated stem cells, progeny thereof, or combinations thereof into the gastrointestinal [organ] alimentary tract of the subject suffering from a gastrointestinal disorder.

22. The method of claim 21 wherein the stem cells are selected from the group consisting of multipotent stem cells, pluripotent stem cells, totipotent stem cells, and combinations thereof.

23. The method of claim 21 wherein the stem cells are neural stem cells.

24. The method of claim 23 wherein the neural stem cells are derived from embryonic neural tissue, adult neural tissue, or combinations thereof.

25. The method of claim 21 wherein the implanted cells repopulate neurons.

26. (AMENDED) The method of claim [25] 21 wherein the subject suffers from a disorder of the enteric nervous system.

27. The method of claim 26 wherein said disorder of the enteric nervous system is selected from the group consisting of achalasia, Hirschsprung's disease, congenital pyloric stenosis, reflux disease, irritable bowel syndrome, and intestinal pseudo-obstruction.

28. [CANCEL] The method of claim 21 wherein the gastrointestinal organ comprises a solid organ.

29. [CANCEL] The method of claim 28 wherein the solid gastrointestinal organ is the liver, the gall bladder, or the pancreas.

30. [CANCEL] The method of claim 21 wherein the gastrointestinal organ comprises a hollow organ.

31. [AMENDED] The method of claim [30] 21 wherein the [hollow] gastrointestinal alimentary tract comprises [organ is] the mouth, the esophagus, the stomach, or the bowels.

32. [AMENDED] The method of claim 21 wherein implanting cells into a gastrointestinal [organ] alimentary tract of a subject comprises administering the cells locally.

33. The method of claim 32 wherein administering cells locally comprises injecting the cells into a wall of the gastrointestinal tract.

34. (AMENDED) A method of repopulating neurons within the gastrointestinal alimentary tract of a subject suffering from a gastrointestinal disorder comprising implanting isolated neural stem cells, progeny thereof, or combinations thereof into the gastrointestinal alimentary tract of the subject suffering from a gastrointestinal disorder.

35. (AMENDED) A method of providing a neurotransmitter within the gastrointestinal alimentary tract of a subject suffering from a gastrointestinal disorder comprising implanting isolated neural stem cells, progeny thereof, or combinations thereof into the gastrointestinal alimentary tract of the subject suffering from a gastrointestinal disorder.

36. The method of claim 35 wherein the neurotransmitter is nitric oxide.

37. The method of claim 35 wherein the neurotransmitter is substance P.

38. (AMENDED) A method of producing nitrinergic neurons in a subject suffering from an enteric nervous disorder comprising implanting isolated neural stem cells into the smooth muscle of the gastrointestinal alimentary tract of the subject suffering from an enteric nervous disorder.

39. The method of claim 38 wherein the neural stem cells are implanted into the pylorus.

40. The method of claim 38 wherein the neural stem cells are implanted into the deuodenum.

41. (AMENDED) A method of treating a disorder of the enteric nervous system in a subject suffering from an enteric nervous disorder comprising implanting isolated neural stem cells, progeny thereof, or combinations thereof into [a] the gastrointestinal alimentary tract [organ] of the subject suffering from an enteric nervous disorder.

42. The method of claim 41 wherein the implanted cells produce nitric oxide.

43. The method of claim 41 wherein the cells are implanted into the pylorus.

44. (NEW) The method of claim 38 wherein said disorder of the enteric nervous system is selected from the group consisting of achalasia, Hirschsprung's disease, congenital pyloric stenosis, reflux disease, irritable bowel syndrome, and intestinal pseudo-obstruction.

45. (NEW) The method of claim 41 wherein said disorder of the enteric nervous system is selected from the group consisting of achalasia, Hirschsprung's disease, congenital pyloric stenosis, reflux disease, irritable bowel syndrome, and intestinal pseudo-obstruction.

PATENT
Docket No. 265.00090101

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s):	Pasricha et al.	Group Art Unit:	1632
Serial No.:	09/834,110	Examiner:	Joseph T. Woitach
Confirmation No.:	5306		
Filed:	April 12, 2001		

For: TREATMENT OF DISORDERS BY IMPLANTING STEM CELLS AND/OR PROGENY THEREOF INTO GASTROINTESTINAL ORGANS

APPOINTMENT OF ASSOCIATE ATTORNEY

Assistant Commissioner for Patents
Mail Stop AF
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

The undersigned attorney of record in the above-identified patent application hereby appoints: Nancy A. Johnson, Attorney Registration No. 47,266, to act as an associate agent, and empowers the associate agent to receive all correspondence from the U.S. Patent and Trademark Office, to amend the specification and drawings, to prosecute the application; and ratifies any act done by the associate agent in respect of the application or any related application thereto.

Respectfully submitted for
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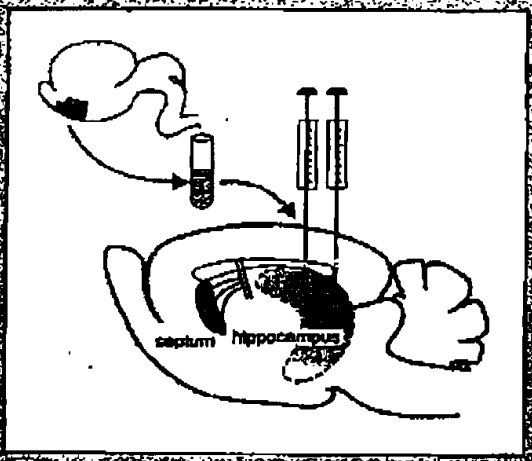
CERTIFICATE UNDER 37 CFR §1.8:
The undersigned hereby certifies that this paper is being transmitted by facsimile in accordance with 37 CFR §1.6(d) to the Patent and Trademark Office, addressed to Assistant Commissioner for Patents, Mail Stop AF, P.O. Box 1450, Alexandria, VA 22313-1450, on this 25th day of July, 2003, at 2:30pm (Central Time).

By: Jill R. Aguilar
Name: JILL R. AGUILAR

Exhibit A

Neural Transplantation Methods

Edited by
Stephen B. Dunnett
Alan A. Boulton
Glen B. Baker



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NEUROMETHODS • 36

Series Editors: Alan A. Boulton and Glen B. Baker

Contents

Immunobiology of Neural Xenotransplantation

Walter C. Low, Wei-Ming Duan, C. Dirk Keene, Hsiao-Tzu Ni, and Marcus A. Westerman

1. INTRODUCTION

The field of neural transplantation has rapidly progressed during the past two decades. Since the first published observations on the functional effects of transplanted fetal dopamine (DA) neurons in rodents (Perlow et al., 1979; Björklund and Stenevi, 1979), there are now several ongoing clinical trials to assess the efficacy of transplanting human fetal neurons for the treatment of patients with Parkinson's disease (PD) (Freed et al., 1992; Widner et al., 1992) and Huntington's disease (HD) (Kopyov et al., 1996). However, a major limitation to the widespread clinical application of this approach is the inability to obtain consistently suitable specimens for transplantation, and the ethical concerns of using human fetal tissue. Consequently, the use of donor tissue from other species as xenografts has been proposed as an alternative to the use of human fetal allografts.

Major barriers exist, however, in the development of neural xenotransplantation. In the transplantation of whole organs between disparate animal species, transplants can be rejected within minutes to hours (hyperacute rejection [HAR]), within hours to days (acute rejection), or within weeks to months (delayed/chronic rejection). The mechanisms involved in the rejection process vary with the type of tissue that is transplanted and the site of transplantation. This chapter presents what is currently known about the major issues concerning the immunobiology of xenotransplantation, and how it relates to the transplantation of neural tissue between species.

2. XENOGRAFT REJECTION

The major obstacles to xenotransplantation are the presence of antibodies (Abs) and cells of the immune system that recognize antigens (Ags) from another species. Natural Abs recognize carbohydrate determinants of the ABO blood group system, and the degree of reactivity of these natural Abs by the host depends on the phylogenic relationship with the donor. Species

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Edited by: S. B. Dunnett, A. A. Boulton, and G. B. Baker. © Humana Press Inc., Totowa, NJ

combinations that result in highly reactive natural Ab responses are said to be discordant. In contrast, species combinations in which the host does not produce natural Abs that recognize donor carbohydrate determinants are said to be concordant. Humans and Old World monkeys are, therefore, thought to be concordant, and humans and pigs are discordant. Natural Abs that establish the degree of species concordance and cellular elements of the host immune system play major roles in the various types of rejection observed within the context of xenotransplantation.

2.1. *Hyperacute Rejection*

Hyperacute xenograft rejection is a complement-mediated reaction involving host Abs binding to xenogeneic Ags. A major source of xeno-Ags are glycoproteins, particularly the Galα1,3Gal terminal oligosaccharide residue(s) (Cooper, 1998; Hammer et al, 1998). Pigs and almost all other mammals (excluding humans and Old World monkeys) express this epitope. Humans express ABO histo-blood-group moities. In hyperacute xenograft rejection, host complement is activated, and the donor vascular endothelium is converted from an anticoagulative to a procoagulative state. As a result, endothelial cells (ECs) undergo swelling, vesiculation, cell–cell detachment, and lysis, which lead to disruption of the endothelial barrier (Saadi and Platt, 1998). Subsequent fibrin deposition, microvascular thrombosis, interstitial edema, and hemorrhage ultimately result in xenograft failure, with minimal cellular infiltration (Hammer et al., 1998). HAR is typically observed in whole-organ xenografts, in which the vasculature of the donor organ is in constant contact with xenoreactive Abs in the host serum.

Cellular transplants, such as those derived from neural and pancreatic tissue, initially lack established graft vasculature, and therefore do not experience HAR (Auchincloss and Sachs, 1998). However, Galα1,3Gal and other potential xeno-Ags are expressed by many cell types, and may have some role in humorally mediated acute and chronic neural xenograft rejection. However, the role of xeno-Ags in cellular xenograft rejection remains to be elucidated.

2.2. *Acute Rejection*

Delayed forms of xenograft rejection, including rejection of tissue from a concordant donor, are mediated through a number of pathways involving Ab- and cell-mediated mechanisms (Dorling and Lechler, 1994). These are very different from those involved in HAR. Acute vascularized allograft and xenograft rejection exhibits less-fulminant endothelial injury, characterized by endothelial swelling, focal ischemia, and diffuse thrombosis (Saadi and Platt, 1998). Furthermore, unlike HAR, acutely rejecting tissues

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If the humoral immune response can be overcome, acute graft rejection is mediated primarily by cytolytic lymphocytes, which depend on interaction with Ag-presenting major histocompatibility complex class I (MHC-I) molecules (Roslin et al., 1992). One of the principle findings in neural xenograft rejection has been a direct correlation between xenograft rejection and MHC upregulation in host and donor tissues (Mason et al., 1986). MHC upregulation stimulates activation of the efferent arm of the immune system, resulting in cell-mediated cytotoxicity and graft rejection (Isacson and Breakefield, 1997).

2.3. *Delayed and Chronic Rejection*

Xenograft rejection has been characterized as chronic, if it occurs over a period of weeks to months. Studies of neural grafts consisting of cell suspensions have noted that, without immunosuppressive agents, the transplanted tissue can survive for a prolonged period of time (Björklund et al., 1982; Low et al., 1985; Daniloff et al., 1985). Neural xenografts, however, are typically rejected over the course of several weeks following transplantation (Duan et al., 1995). The humoral arm of the immune response might play a role, albeit less than with HAR, as the result of the development of donor vascular endothelium. However, the blood–brain barrier (BBB), once reestablished, will significantly limit humoral access to grafted neurons and glia. Cell-mediated mechanisms are clearly involved. Infiltration of $CD4^+$ and $CD8^+$ cells of the host have been noted within the graft site (Duan et al., 1995). This observation is consistent with increased T-cell infiltration seen in chronically rejecting xenogeneic cartilage transplants (Stone et al., 1997).

3. MECHANISMS OF GRAFT REJECTION

Xeno-Ag presentation mediates graft rejection, and involves both the afferent and efferent arms of the immune response. In acute graft rejection, for example, prior to reestablishment of the BBB, humoral responses (Ag–Ab-dependent) may be more likely to cause rejection, while chronic rejection paradigms probably rely more heavily on cell-mediated (MHC–T-cell-receptor [TCR]-dependent) cytotoxicity. The following sections contain discussions of mechanisms of humoral and cell-mediated immunity, evidence for their involvement in neural xenograft rejection, and strategies for their suppression.

3.1. *Humoral Response to Xeno-Ag*

The humoral immune response is Ab-mediated. B-lymphocyte membrane immunoglobulin (Ig)-bound peptide, carbohydrate, or lipid initiates B-cell activation through a tyrosine kinase-dependent intracellular signaling pathway.

Activated B-cells upregulate MHC-II expression, resulting in increased activation of T-helper (Th)-cells (via MHC class II Ag presentation and interaction of co-stimulatory molecules B7 and CD40). Once activated, Th-cells release specific cytokines that stimulate B-lymphocyte proliferation, Ig secretion, isotype switching, and memory cell formation, as well as leukocyte and monocyte activation and migration to activated areas (such as lymph nodes and graft sites).

Th activation is greatly decreased in response to nonpeptide Ag, because MHC-II molecules cannot present carbohydrate or lipid moieties to $CD4^+$ cells. However, the humoral immune response to the carbohydrate xeno-Ag Galα1,3Gal, is highly pronounced. The vigorous host immune response to these epitopes is explained by efficient carbohydrate Ag–Ab-mediated B-cell crosslinking, resulting in very strong B-lymphocyte activation and proliferation, and by Ag–Ab complex-induced complement activation described in detail in Section 3.3.).

The humoral response to neural xeno-Ags is probably most pronounced early, following engraftment resulting from disruption of the tight endothelial barriers of the central nervous system. Support for this comes from the demonstration that early xenorecognition leads to differential activation of Th2-specific lymphocytes, which primarily mediate the humoral arm of the immune system (Wren et al., 1993). However, skin-graft-induced rejection of long-surviving islet xenotransplants is also mediated primarily by Th2-cells, suggesting humoral involvement in chronic rejection paradigms as well (Morris et al., 1995). The role of the humoral response in neural xenograft rejection is not clear, and studies in other systems may not be applicable because of the immunologically privileged nature of these neural transplants.

3.2. *Natural Xeno-Abs*

Naturally occurring Abs to xenogeneic Ags are produced in mammals without prior exposure and sensitization. They exist perhaps as a result of bacterial colonization of the gastrointestinal tract, and are mostly directed toward carbohydrate epitopes. These natural Abs then crossreact to similar epitopes on xenogeneic tissue. The principle human natural xeno-Ab is IgM, directed toward the Galα1,3Gal epitope. However, characterization of the humoral response to this epitope has almost exclusively been in vascular endothelium during HAR. Since fetal neural xenografts contain little vascular endothelium, and may not express Galα1,3Gal at early developmental time-points, the classic HAR exhibited in mature solid organ xenografts does not occur. However, the BBB is disrupted for days to weeks following engraftment, allowing any naturally occurring host xeno-Abs free access

to the graft site, and severity of graft rejection is directly correlated with the degree of BBB compromise (Nakashima et al., 1988).

Neural allo- and xenografts develop a chimeric vasculature as they mature, consisting of host pia-derived and graft-derived vascular endothelium, with donor endothelium comprising the majority of the vasculature (Krum et al., 1988; Kobsaka et al., 1989). Studies by Geist et al. (1991) and Rosenstein et al. (1992) showed that the BBB in neural allo- and xenotransplants is competent at two weeks posttransplantation, indicating that traditional HAR mechanisms cannot occur in neural xenotransplants. However, eventual development of graft-derived endothelium results in significant endothelial xeno-Ag exposure to host serum.

IgM xeno-Abs have limited access to grafted cells beyond the endothelial barrier of the vasculature. However, Hulsebosch and Fabian (1989) showed that rabbit intraperitoneal (ip)-injected IgG penetrated all regions of the neuraxis parenchyma 48 hours after injection. Thus, xeno-Ags expressed on grafted neurons and glia may be important, particularly with regard to IgG-mediated cytotoxicity. The identity of these prospective xeno-Ags is undetermined, although the swine leukocyte antigens may be important for porcine xenograft rejection (Cooper, 1998). It will also be of interest to examine xeno-Ab composition in anti-Galα1,3Gal Ig-depleted animals receiving neural xenografts. Although characterization of the repertoire of natural xeno-Abs directed toward neural grafts is in its infancy, it may be possible to produce donor animals with low xeno-Ag expression by selectively breeding animals exhibiting low xeno-Ab adsorption (Alvarado et al., 1995).

3.3. Complement and HAR

The complement system provides many of the effector functions of the humoral immune system, and is the key player in hyperacute vascular xenograft rejection. Complement mediates the activation of inflammation, opsonization, and cytolysis, and positively regulates the humoral response. Activation of complement, via classical (IgG- or IgM-dependent) or alternative (Ig-independent) pathways, results in the formation of complement enzyme fragments, which mediate anaphylaxis and chemotaxis, and membrane attack complexes (MAC), which lyse cells. Partial MAC (C5b67) may also be important in disrupting intercellular endothelial adhesion, leading to endothelial membrane disruption. The complement cascade leads to endothelial failure and graft rejection directly (MAC, C5b67) and indirectly (T-lymphocyte and macrophage infiltration and activation).

The complement cascade is tightly controlled through regulators of complement activation (RCAs). Principal among these are Factor I (and its co-factors)

and decay-accelerating factor (DAF), which affect the inactivation/degradation of complement cascade enzymes. However, xenogeneic RCAs tend functionally not to cross species barriers. For this reason, and because of the influence of natural xeno-Abs, complement-mediated graft rejection is particularly intense. As a consequence, RCAs, primarily DAF, have become targets of modulation for immunosuppression in organ xenotransplantation (*see* Section 12.1.).

Little is known about complement-mediated neural xenograft rejection, and classic hyperacute graft rejection is not observed in neural xenografts. Although αGal-mediated EC activation can occur in the absence of complement (Palmetshofer et al., 1998a,b), whole-organ xenotransplant rejection is mediated principally via the complement cascade. Multiple novel approaches are being tested to inhibit this cascade of events leading to xenorejection. These include removing Galα1,3Gal-reactive Abs, using plasmapheresis/affinity columns (Besse et al., 1994); blocking Galα(1,3Gal Abs by administering Ag or an anti-idiotype Ab (Sandrin and McKenzie, 1994); removing the Galα1,3Gal Ag, using knock-out donors (Tearle et al., 1996), enzymatic treatment (Sandrin and McKenzie, 1994), altered expression through promoter modification (Sandrin and McKenzie, 1994), altered expression using antisense oligonucleotides (d'Apice and Pearse, 1996), and epitope masking with other carbohydrates such as sialic acid (Sandrin and McKenzie, 1994); inducing tolerance to xeno-Ags using retroviral transfer of xeno-Ab synthetic machinery to bone marrow cells (Bracy et al., 1998); and inhibiting complement by using donor tissue transgenic for human complement regulator proteins (DAF, membrane cofactor protein [MCP], CD59) (Hammer et al., 1998). Concerning the latter approach of modifying complement regulatory proteins, recent studies have examined the effect of transplanting ventral mesencephalon (VM) cells from transgenic fetal pigs that overexpress human CD59 into cyclosporin-A (CsA)-treated rats with 6-hydroxydopamine (6-OHDA) lesions (Deacon et al., 1998). Rats treated with transgenic CD59 porcine cells exhibited an amelioration of amphetamine-induced rotational bias. Because the porcine cells were transfected with cDNA that encodes human CD59 protein, it is not known whether this genetic manipulation would have any consequence on graft survival in the host rat. However, this study does demonstrate the functional nature of the transgenic cells with respect to rectifying neurologic deficits.

4. CELL-MEDIATED XENOREACTIVITY

Inhibition of humoral-mediated xenograft rejection in experimental models of discordant xenotransplantation has revealed the involvement of

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cell-mediated mechanisms. When the hyperacute immune response is blocked, xenograft rejection occurs following infiltration by macrophages, natural killer (NK) cells, and lymphocytes, in a fashion similar to that seen in allograft rejection (Auchincloss, 1988). Indeed, the mechanisms of cellular-mediated immunity to xenografts are fundamentally similar to those involved in allograft rejection (Yamada and Der Simonian, 1995). However, cell-mediated xenograft rejection cannot be controlled by the types of nonspecific immunosuppression used routinely to prevent allograft rejection. Thus, although fundamentally similar, the cellular responses in xenotransplantation may differ somewhat mechanistically from those in allotransplantation. The following sections focus on mechanisms of cell-mediated rejection in xenotransplantation, and on differences between cell-mediated xeno- and allotransplant rejection. An understanding of these differences may provide more focused and specific approaches to control the rejection of xenografts.

4.1. Presentation of Xeno-Ags

Cell-mediated graft rejection is dependent on specific signaling between the various cell types involved. The first step in this process is the recognition of graft Ags as nonself. This occurs through the presentation of xeno-specific epitopes, and is dependent on the nature of the xeno-Ag, cellular and molecular phenotype of both presenting and recognizing cells, and cytokine disposition.

MHC is required for xeno-Ag presentation. MHC molecules bind innate and foreign peptides, and are then expressed as MHC–Ag complexes on the cell surface, for sampling by T-lymphocytes through TCRs. Neurons do not express MHC-II, and normally express very low levels of MHC-I, unless stimulated by cytokines during processes such as inflammation (Pakzaban and Isacson, 1994). Although neurons can be induced to express MHC-I, other graft-derived cells probably mediate xeno-Ag-specific rejection. Donor microglia can function as antigen-presenting cells (APCs), and may participate, but this is unlikely, because microglia transplanted within neural cell suspensions are limited in number from the outset, and are rapidly replaced by those of the host rat (Geny et al., 1995). Glial cells may provide cues for recognition by the host immune system, but grafts composed predominantly of astrocytes do not induce rejection (Pollack et al., 1992). The exact role of specific cell types in neural xeno-Ag presentation remains to be determined.

4.2. Ag–MHC–TCR Interactions

Ag presentation is normally mediated by MHC–peptide interactions with TCR. There are two classes of MHC, which serve different functions. MHC-I

molecules are expressed on all nucleated cells, and normally present peptide Ags that are intrinsic to the cytoplasm. In contrast, MHC-II is expressed on cells that are specialized for Ag presentation. These so-called APCs include B-lymphocytes, macrophages, and dendritic cells, and normally present Ag that has been endocytosed from the extracellular space. T-lymphocyte CD4 and CD8 cell surface molecules are co-receptors for the TCR, and restrict TCR interactions to MHC-II and -I, respectively. $CD4^+$ Th-cell activation, via binding with the MHC-II–peptide complex, results in cytokine production that stimulates leukocyte and monocyte activation, and migration to activated areas (such as lymph nodes and graft sites). $CD8^+$-cell activation, via binding with the MHC-I–peptide complex, results in $CD8^+$ T-cell mediated cytotoxicity. The MHC phenotype of a graft can therefore dramatically influence its survival.

There is abundant evidence supporting the involvement of MHC in neural xenograft rejection. Pollack et al. (1990) demonstrated that, although histological features of neural xenotransplant rejection varied, depending on the method of induction (i.e., skin graft, contralateral eye removal, and mannitol BBB disruption), upregulation of MHC was prevalent in all experimental manipulations examined. Also, murine $MHC\text{-}I/II^+$ corpus collosum xenotransplants are rejected, but $MHC\text{-}I/II^-$ astrocytic cell lines are not (Litchfield et al., 1997), and cultured porcine neurons (MHC^+) exhibit a much stronger proliferative T-cell response than freshly isolated porcine neural tissue (MHC^-) (Brevig et al., 1997). These studies support the notion that donor MHC expression is necessary for fulminant graft rejection. However, the severity of the response may depend in part on the mechanism of Ag presentation.

4.3. Direct vs Indirect Ag Presentation

The nature of xeno-Ag presentation is controversial. Xeno-Ags may be presented either directly or indirectly to activate the immune system. Direct Ag presentation involves the direct recognition of donor MHC molecules by host cells, i.e., donor MHC molecules are able to bind the TCR of host T-cells and stimulate them directly. Indirect Ag presentation, on the other hand, involves the recognition by host TCR of host MHC containing donor peptide. Allografts, by definition, can present Ag by both mechanisms. However, the ability of a xenograft to present Ag directly to the host immune system is highly species-combination-specific.

Human xeno-Ag can be presented directly and indirectly to murine T-cells. Kievits et al. (1989, 1990), using MHC transgenic mice, found that xeno-MHC-specific (anti-B27) mouse cytotoxic T-lymphocytes (CTLs) could kill

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human and mouse cells expressing the appropriate xeno-Ag in a self-MHC unrestricted manner. Mixed lymphocyte reaction (MLR) studies have shown that human leukocyte antigen (HLA)-G functions both as a xeno-Ag (indirect), and as a restriction element that can present peptides to murine T-cells (direct) (Horuzsko et al., 1997). Bluestone et al. (1987) provided evidence for direct Ag presentation by porcine MHC to mouse TCR. They demonstrated that mouse splenocytes transgenic for porcine MHC-I molecule stimulated normal spleen cells to generate MHC-I-specific CTL, showing that mice have an immunological repertoire specific for xenogeneic class I gene product.

In vivo studies provide further support for species combination specificity in Ag presentation. In a human-to-mouse paradigm, transgenic HLA-G murine tissue was rejected by host mice, either directly or as a peptide in the context of murine MHC (Horuzsko et al., 1997). Furthermore, by masking donor human HLA-G, liver and islet xenograft survival were prolonged. Support for pig-to-rat direct Ag presentation comes from studies that demonstrated that masking of donor porcine striatal MHC-I cells with anti-porcine MHC monoclonal antibody (mAb) substantially enhanced survival of xenografts (Pakzaban et al., 1995).

There is mixed support for direct Ag presentation in rat-to-mouse combinations. Hirota et al. (1997), using MLR, showed that mouse T-cells recognize rat xeno-Ags via both direct (xeno-APC-restricted) and indirect (self-APC-restricted) mechanisms, and that CD4+ and CD8+ T-lymphocytes participate in the direct pathway. However, Kano et al. (1998) showed that treatment with rat-antimouse mAb to APC expressing B7-1 and B7-2 co-stimulatory molecules inhibited antidonor Ab formation and deposition of C3, IgM, and IgG on xenograft rat cardiac endothelium, but mouse-antirat mAb had little effect, suggestive of an indirect Ag presentation paradigm.

These studies indicate that there is direct Ag presentation in xenotransplants between some species. Direct Ag presentation is important in neural transplantation, because donor neural tissue MHC has been shown to be upregulated sooner than the MHC of the surrounding parenchyma (Litchfield et al., 1997). Therefore, direct Ag presentation may lead to a more vigorous immune response to neural xenografts.

Extensive preclinical testing of a specific species for xenotransplantation is not advisable unless it exhibits minimal direct Ag presentation to human T-lymphocytes. In order to minimize direct Ag presentation, selection of appropriate donor tissue for clinical applications must be done carefully. MLR testing of species-specific combinations will be a valuable tool in this regard. The outlook for porcine xenografting, in this respect, is problematic.

Human T-lymphocyte responsiveness to porcine endothelium is very strong, and is mediated in part by direct Ag presentation (Binmele et al., 1996; Murray et al., 1994). However, using donor tissue from animals that exhibit genetically modified MHC expression (e.g., MHC knock-out pigs) can, theoretically, circumvent this problem.

The use of MHC knock-out donor tissue is currently being evalnated. These studies have found that MHC-deficient mouse-to-rat xenograft survival is not enhanced in vascularized organ transplantation (Markmann et al., 1994; Chitilian and Auchincloss, 1997), although donor MHC-I and -II knock-out neural tissue has shown markedly increased graft survival with cellular transplants (Duan et al., 1998).

4.4. *Sensitivity and Specificity of Xeno-Ag Presentation*

MHC–TCR interactions may be less efficacious in xenotransplantation, because of decreased host TCR recognition of donor MHC, and decreased co-stimulatory molecule homology. There is evidence to suggest, however, that this may not be the case. Gress et al. (1989) showed that xenogeneic Ag recognition by human T-cells is very specific, can be altered with single amino acid changes, and is comparable to the specificity of MHC-complex-encoded molecule differences that are recognized in allogeneic paradigms by murine CTL. Furthermore, xenogeneic co-stimulatory molecules may contain sufficient homology with host molecules to interact as though they were host-derived. Maher et al. (1996) provided evidence to support this notion, by demonstrating that porcine ECs express CD86, which strongly co-stimulates human T-cells through CD28. They showed that co-stimulation of human T-cells by chinese hamster ovary (CHO) or human umbilical cord vascular endothelial cells, transfected with porcine CD86, is as effective as co-stimulation by human CD80 or CD86. The degree of molecular interaction between species, therefore, will depend on the specific species involved.

5. T-LYMPHOCYTES

Although alloreactivity is mediated by both $CD4^+$ and $CD8^+$ T-lymphocytes, studies suggest that xenoreactivity is predominantly mediated by $CD4^+$ T-cells. In vitro studies demonstrate that, in response to xeno-Ags, T-cell proliferation and lymphokine production are dependent on $CD4^+$ Th-cells and self-Ag-presenting cells (Moses et al., 1990). Furthermore, in vivo studies of recipients treated with anti-CD4 Abs demonstrate that survival of transgenic mouse skin grafts expressing porcine class MHC-I is prolonged, compared to anti-CD8-treated and untreated controls (Auchincloss et al., 1990). Other in vivo depletion experiments and adoptive transfer studies

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Similar lymphocyte-depletion studies in neural transplant models have shown that CD4+ T-lymphocytes are essential for initiating the rejection of xeno- and allogeneic CNS grafts, but CD8+ T-lymphocytes are unable by themselves to mediate rejection (Nicholas et al., 1990; Wood et al., 1992). Together, those studies indicate that CD4+ Th-cells are critical in neural xenograft rejection, and that foreign Ags must be presented to the Th-lymphocytes by MHC-II-expressing cells.

6. CYTOKINE RESPONSES

CD4+ Th1-cells, which produce primarily IL-2 and interferon γ (IFN-γ), predominate in cell-mediated responses, while CD4+ Th2-cells, which produce primarily IL-4 and IL-10, predominate in humoral cellular responses (Mosmann et al., 1986, 1991; Salgame et al., 1991). Th2 cytokines (IL-10) crossregulate Th1 activity (IFN-γ) and vice versa (Fernandez-Botran et al., 1988; Fiorentino et al., 1991; Mosmann et al., 1991). The cytokine profile of CD4+ T-cells in response to xenografts is important, to better understand their role in xenoreactivity. In a one-way MLR, Wren et al. (1993) showed that xeno-Ag-stimulated T-lymphocytes predominantly exhibited a Th2-type cytokine response, in contrast to the Th1 response of allo-Ag-stimulated lymphocytes. Preferential Th2 responsivity to xenografts was also found in islet transplantation studies (Morris et al., 1995).

If the Th2 response predominates in xenoreactivity, it may explain why conventional immunosuppressive agents, which are successful in prolonging allograft survival, are less efficacious in preventing xenorejection. However, studies using various cytokine knock-out mice have not revealed specific Th2 cytokines that are essential for xenograft rejection. Thus, differences in cytokine response to allo- and xenotransplantation remains to be clarified. A better understanding of the balance between Th subset function and cytokine profile in neural xenotransplantation may allow more targeted and specific approaches to control the early events in rejection.

7. NK CELLS

NK cells are believed to participate in xenograft rejection. MHC-I molecules contain specific motifs that inhibit NK cell-mediated cytotoxicity, but xenograft MHC-I inhibitory domains may not be recognized by host NK cells (Moretta, 1996). Thus, lack of appropriate MHC-restricted Ag pres-

entation can induce graft rejection, which is a concern for MHC knock-out xenotransplantation. In addition, NK cells can be activated through the recognition of cell-surface-bound IgG, resulting in induction of Ab-dependent, cell-mediated cytotoxicity, and release of cytokines, such as tumor necrosis factor α (TNF-α) and interferon-γ (IFN-γ).

There is clear evidence that NK cells participate in xenogeneic rejection. Human NK cells have been shown to activate and lyse ECs by direct cell contact, when co-cultured with porcine ECs (Goodman, 1996). In vitro studies show that NK inhibitory receptors fail to bind to xenogeneic MHC-I molecules (Bezouska et al., 1994; Ballas et al., 1996), and that transgenic porcine ECs expressing human HLA subtypes are less susceptible to human NK cell-mediated cytotoxicity than are controls. Furthermore, multiple MHC-I subtypes need to be expressed to inhibit polyclonal NK cell populations, demonstrating the existence of multiple MHC inhibitory motifs that are recognized by distinct classes of NK cells (Seebach et al., 1997).

In vivo studies have shown a mononuclear infiltrate consisting of monocytes and NK cells associated with xenograft rejection (Inverardi et al., 1992; Hancock et al., 1993). Depletion of NK cells, in combination with other forms of immunosuppression, prolongs graft survival (Umesue et al., 1996). However, additional NK cell depletion has no additive effect on xenograft survival resulting from $CD4^+$ T-cell depletion (Karlsson-Parra et al., 1996), suggesting a T-cell participation in NK-mediated xenograft rejection. The role of NK cells in xenotransplantation at immunologically privileged sites, such as the brain, remains to be elucidated.

8. CO-STIMULATORY/ADHESION MOLECULES

Adhesion molecules are important for the binding and extravasation of leukocytes to/through activated endothelium, and for the cell–cell interactions that mediate immune activation and cytotoxicity. Co-stimulatory molecules, which include many adhesion molecules, are important for T-cell activation during MHC–TCR interaction. The following section focuses on functional aspects of these molecules.

8.1. Adhesion Molecules

Adhesion molecules function through specific binding to other cells (via specific interactions with other adhesion molecules) or to extracellular matrix. Leukocytes and other cells use these interactions to adhere to activated endothelium, thereby promoting extravasation into an immunologically stimulated region, such as a xenograft site. Specific adhesion molecule expression determines the types of cells that invade a graft, and organ-spe-

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cific and vessel endothelium-specific differences have been demonstrated (Steinhoff et al., 1994). Vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), and lymphocyte function associated antigen-1 (LFA-1) are particularly important in xenograft rejection. Blocking VCAM-1 results in decreased binding of human T-cells to activated porcine endothelium (Mueller et al., 1995); mAb specific for the LFA-1 binding epitope of ICAM-1 increased islet xenograft survival, and delayed lymphocyte infiltration into the graft (Zeng et al., 1994). LFA-1 is also prominently involved in NK cell adhesion to activated endothelium (Inverardi et al., 1992). Significant evidence supports the role of ICAM-1–LFA-1 and VCAM-1–CD49 in promoting leukocyte activation and infiltration regarding xenografts (Ohta et al., 1998; Kwiatkowski et al., 1998), but little evidence exists concerning cell adhesion molecule roles in neural xenograft rejection.

Antineural xenograft rejection therapies are being investigated, using mAb to specific adhesion molecules with some efficacy (*see* Section 11.4.). Pharmacological intervention may also prove useful. For instance, Lin et al. (1998) have previously shown that a 2-wk course of leflunomide, which suppresses inducible E- and P-selectin expression, together with maintenance cyclosporin A (CsA) therapy, rendered hamster-to-rat cardiac xenografts resistant to antihamster IgM xeno-Ab-mediated rejection (xenograft accommodation). The authors also demonstrated that constitutive adhesion molecule expression, such as ICAM-1, is downregulated in such an accommodated graft.

8.2. Co-Stimulatory Molecules

Activation of T-cells is initiated by specific TCR recognition of MHC-bound Ags. An interaction of accessory molecules between T-cells and APCs is necessary to provide the co-stimulatory signal(s) for complete T-cell activation. Co-stimulatory molecule interactions are neither MHC-restricted nor Ag-specific, and include the B7 family (interacting with CD28/CTLA-4) (Gimmi et al., 1991; Linsley et al., 1991; Harding et al., 1992; Freeman et al., 1993; Harding and Allison, 1993; Linsley and Ledbetter, 1993), ICAM-1 (interacting with LFA-1 [β_2-integrin]) (Seventer et al., 1990; Kuhlman et al., 1991), and VCAM-1 (interacting with VLA-4 [β_1-integrin]) (Damle and Aruffo, 1991; Damle et al., 1992). Other important co-stimulatory molecule relationships include LFA-2 (CD2) interacting with LFA-3, and CD44 and L-selectin interacting with endothelium and cell matrix. Co-stimulatory molecules can enhance or suppress T-cell activation, and expression of these molecules is highly regulated. The specific role of co-stimulatory molecules in neural xenograft rejection remains to be determined.

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9. BRAIN AS IMMUNOLOGICALLY PRIVILEGED SITE FOR TRANSPLANTATION

Xenograft rejection is greatly influenced by the site of transplantation. The brain has long been considered an immunologically privileged site. Histoincompatible (allo- or xeno-) grafts survive longer in the brain than grafts placed in peripheral sites (Barker and Billingham 1977; Head and Griffin, 1985; Widner and Brundin, 1988). Several factors contribute to the immunologically privileged status of the brain, including the presence of a BBB (Barker and Billingham 1977); the lack of a conventional lymphatic drainage system (Bradbury and Westrop, 1983; Head and Griffin, 1985); the absence of APCs, particularly dendritic cells (professional APC) (Hart and Fabre, 1981); very low or no MHC expression on brain cells (Lampson, 1987; Skoskiewicz et al., 1985; Wong et al., 1984, 1985); and the existence of local immunosuppressive factors (Jackson et al., 1987; Massa, 1993; Wilbanks and Streilein, 1992).

9.1. *Blood–Brain Barrier*

The BBB exists because normal brain capillaries contain specialized elements that enable them to tightly regulate the passage of molecules and cellular elements between the blood and the brain. The BBB consists of microvascular ECs in close apposition with abluminal astrocytic foot processes.

Alteration or destruction of the BBB can occur during a variety of pathological conditions, including brain trauma (Dietrich et al., 1994; Schmidt and Grady, 1993; Soares et al., 1995), hemorrhage (Germano et al., 1992; Smith et al., 1996), and certain nervous system diseases, e.g., experimental allergic encephalitis (EAE) (Paul and Bolton, 1995), multiple sclerosis (Brown and Dumonde, 1986), tumors (Grabb and Gilbert, 1995; Roelcke et al., 1995), Alzheimer's disease (Kalaria, 1992), and hypertension (Tang et al., 1993). The BBB is compromised for macromolecules up to 1 wk following neural transplantation (Bertram et al., 1994; Broadwell, 1990; Broadwell et al., 1991; Brundin et al., 1989). The time during which the BBB is opened may vary, depending on the type of neural graft and the immunological status of the recipient. Consequently, during this period, increased intraparenchymal leukocyte and Ab infiltration may lead to xenograft rejection.

9.2. *Lack of Conventional Lymphatic Drainage System in Brain*

The brain lacks a lymphatic drainage system that is comparable to other tissue systems in the body. However, a large number of studies have shown that foreign cells and Ags placed in the brain are subsequently transported to lymphoid organs, such as deep cervical lymph nodes or the spleen (Bradbury

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and Westrop, 1983; Broadwell et al., 1994; Csorr et al., 1992; Kida et al., 1993; Weller et al., 1992; Widner et al., 1988; Yamada et al., 1991). Recently, Broadwell et al. (1994) observed that donor-derived MHC-I-positive cells from intracerebral neural allografts can be identified immunocytochemically in the host spleen and lymph nodes. Possible pathways from the brain to lymph organs include the subarachnoid space and drainage of cerebral extracellular fluids (e.g., cerebrospinal fluid) through the cribiform plate into the cervical lymph (Harling-Berg et al., 1989; Kida et al., 1993; Szentistvanyi et al., 1984; Weller et al., 1992; Widner and Brundin 1988). These studies support the existence of pathways between the brain and immune organs for the trafficking of APCs that can initiate the afferent arm of xenograft rejection.

9.3. Expression of MHC Ags in Brain

Normally, brain cells do not express detectable levels of MHC molecules (Lampson, 1987; Skoskiewicz et al., 1985; Wong et al., 1984, 1985), but certain cells in the brain can be induced to express MHC molecules. These include ECs (Finsen et al., 1991; Linke and Male, 1994), ependymal cells (Steiniger and van der Meide, 1988), microglia (Bo et al., 1994; Finsen et al., 1991; Panek and Benveniste, 1995; Poltorak and Freed, 1989; Subramanian et al., 1995; Tooyama et al., 1990; Xu and Ling, 1995), and astrocytes (Fierz et al., 1985; Traugott and Lebon). As stated in Section 4.3., MHC expression can also be induced in the host brain following intracerebral neural transplantation (Duan et al., 1993, 1995; Finsen et al., 1991; Isono et al., 1993; Lawrence et al., 1990; Mason et al., 1986; Pollack et al., 1990; Poltorak and Freed, 1989; Wood et al., 1992), and transplantation of neural xenografts normally leads to pronounced expression of MHC Ags within the graft site (Duan et al., 1995; Pollack et al., 1990). These studies suggest potential MHC-mediated interactions between donor and host cells that may lead to graft rejection.

9.4. Absence and Paucity of Ag Presentation Cells in Brain

Dendritic cells (DCs) have the capability of phagocytosis and processing of foreign Ags for presentation in association with MHC-I and -II molecules. These cells are considered to be the most efficient type of APCs for T-cell activation in the body (Lechler and Batchelor, 1982). After DCs take up Ags, they can migrate to the lymphoid organs, where they present Ags to lymphocytes. DCs are found in various tissues in the body, and are represented as Langerhans cells in skin, blood DCs in serum, and interdigitating DCs in lymphoid organs. It is believed that DCs do not exist in the brain (Hart and Fabre, 1981), but some cells in the brain, such as astrocytes,

microglia, and ECs, may be able to function as APCs (Fierz et al., 1985; Finsen, 1993; Finsen et al., 1993a; Poltorak and Freed, 1989; Traugott et al., 1985). In vitro studies demonstrate that cultured microglia, ECs, and astrocytes can be induced to express MHC-I or -II Ags, or both, following exposure to the cytokines IFN-γ or TNF-α (Benveniste et al., 1991; Frei et al., 1987; Hirsch et al., 1983; Massa et al., 1987, 1989; Mauerhoff et al., 1988). Moreover, there is evidence that astrocytes can express MHC Ags in the brain (Traugott et al., 1985). In neural grafting studies, Poltorak and Freed (1989) claimed that host microglia express MHC-II Ags, and concluded that host microglia act as APCs. This finding is further supported by studies in which MHC-II immunoreactive cells in neural implantation sites were found to resemble microglia (Duan et al., 1993, 1995; Finsen 1993; Finsen et al., 1991, 1993). Thus, despite the apparent paucity of APCs within the brain, microglia and astrocytes may be capable of xeno-Ag processing and presentation.

9.5. Existence of Local Immunosuppressive Factors in Brain

Accumulating evidence suggests that several factors in the brain have immunosuppressive effects. Massa (1993) observed that brain gangliosides suppress the expression of both MHC-I and -II molecules on cultured astrocytes, while Jackson et al. (1987) demonstrated the ability of these gangliosides to suppress in vitro human and murine lymphocyte proliferative responses. Cytokines in the brain, such as transforming growth factor-α (TGF-α), IL-10, and IFN-γ, may also exert immunosuppressive effects. Wilbanks and Streilein (1992) found that fluids from immune privileged sites (e.g., cerebrospinal fluid) contain TGF-α, and disrupt the ability of macrophages to present Ags. Recently, IFN-γ, has been found to reduce the Ag-presenting capacity of human glial and B-cells (Jiang et al., 1995), and to downregulate MHC Ag expression (Huynh et al., 1995). In another in vitro study, a soluble inhibitory factor in the CNS was found to inhibit macrophage migration (Hirschberg and Schwartz, 1995). Although these molecules may contribute to the immunologically privileged status of the brain, their exact role requires further clarification.

9.6. CNS Immunological Privilege Is Not Absolute

Accumulating evidence challenges the concept of the brain as an immunologically privileged site. A number of studies have demonstrated that the immunological privilege of the brain is not absolute, and can be abrogated under certain circumstances, such as following orthotopic skin grafts (Freed, 1983; Freed et al., 1988; Nicholas et al., 1987; Rao et al., 1989), following intravenous injections of spleen cells (Mason et al., 1986; Shinoda et al., 1995), or during treatment with ip injections of IFN-γ, (Subramanian et al.,

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1995). Moreover, the immunological privilege of the brain cannot shield neural xenografts from rejection (Brundin et al., 1985a; Finsen et al., 1988a; Honey et al., 1990; Inoue et al., 1985; Nakashima et al., 1988; Pakzaban and Isacson, 1994; Sakai et al., 1991; Wood et al., 1996). As a consequence, methods to inhibit xenorejection within the brain need to be developed.

10. IMMUNOSUPPRESSIVE AGENTS

Immunosuppressive agents act systemically, through various mechanisms, to inhibit the immune response. Intracerebral neural xenografts, similar to other tissue types of xenografts in peripheral sites, are rejected if the recipient animals are not immunosuppressed (Brundin et al., 1985a; Finsen et al., 1988a; Honey et al., 1990; Inoue et al., 1985; Isacson et al., 1995; Nakashima et al., 1988; Pakzaban and Isacson, 1994; Sakai et al., 1991; Wood et al., 1996; Zhou et al., 1993a). This section focuses on immunosuppressive agents that are applicable to neural xenotransplantation.

CsA was first tested as an immunosuppressive drug in neural xenotransplantation in the 1980s (Brundin et al., 1985; Finsen et al., 1988; Inoue et al., 1985), and is still commonly used in evaluating human-to-rodent grafts (Kondoh et al., 1995; Pundt et al., 1996). Long-term, daily administration of CsA is necessary to prevent rejection of neural xenografts (Brundin et al., 1989), but chronic treatment with CsA is accompanied by side effects, such as hepatotoxicity, nephrotoxicity, and gingival hyperplasia, and renders the recipient more prone to infection. In a recent review of the literature, a meta-analysis of several studies revealed that immunosuppression by CsA can only increase the survival rate of intracerebral xenografts from 30 to 74% (Pakzaban et al. 1994). Therefore, alternative immunosuppressive therapies are desirable.

FK506 is similar, both in mode of action and side effects, to CsA (Hoffman et al., 1990; Ochiai et al., 1987). Both CsA and FK506 selectively inhibit calcineurin by binding to the cytoplasmic proteins cyclophilin and FK506-binding protein, respectively (Dawson et al., 1994; Sewell et al., 1994; Parsons et al., 1994). Calcineurin phosphatase activity is blocked, resulting in the inhibition of T-cell activation (Liu et al., 1991). In previous studies, a short-course treatment with FK506 was shown to be effective in preventing rejection of intracerebral neural xenografts (Sakai et al., 1991, 1993). However, FK506 is also nephrotoxic (Nielsen et al., 1995).

Another immunosuppressive drug, 15-deoxyspergualin, is capable of preventing the rejection of intrastriatal neural xenografts (Zhou et al., 1993a), although high toxicity prevents its clinical use. Recently, Duan et al. (1996) showed that a high dose of the corticosteroid, methylprednisolone (MP), can prevent rejection of xenografts as efficiently as CsA, by reducing

T-cell infiltration and cytokine production in the graft site. In addition, MP downregulates cell surface adhesion molecule expression by reducing cytokine production (Cronstein et al, 1992; Sackstein and Borenstein, 1995), resulting in decreased white blood cell adhesion to vascular ECs. Moreover, there is evidence that MP acts as a free radical scavenger, and can reduce lipid peroxidation following spinal cord injury (Anderson et al., 1994; Braughler and Hall, 1981, 1982; Hall and Braughler, 1982; Saunders et al., 1987).

A combination therapy of CsA, the anti-inflammatory steroid, prednisolone, and azathioprine may be more efficacious in prolonging the survival of intracerebral neural xenografts than single immunosuppressive drug treatments. Pedersen et al. (1995) employed this triple-drug regimen to prevent rejection of hippocampal xenografts. This combination is also commonly used clinically in kidney and neural tissue transplantation, and has been shown to be more effective than treatment with CsA alone, enhancing the survival rate to 93% (Pedersen et al., 1995).

The use of specific Abs to block the activity or recognition of immunological components may provide another strategy to prevent neural xenograft rejection. Pakzaban et al. (1995) showed that the use of Abs to mask donor MHC-I resulted in increased survival of pig-to-rat striatal xenografts. Moreover, administration of anti-IL-2 receptor (Honey et al., 1990) or anti-CD4, and -CD8 (Wood et al., 1996) Abs can prevent rejection of neural xenografts.

11. INDUCTION OF TOLERANCE

Currently used immunosuppressive therapies are relatively nonspecific, and must be administered chronically. Ideally, short-term induction of immunosuppression would achieve long-term nonresponsiveness to specific xeno-Ags, without impairing the response to infectious agents. To this end, much research has concentrated on discovering ways to induce specific hyporesponsiveness, or tolerance, across xenogeneic barriers. The following section focuses on several promising approaches to tolerance induction in xenograft transplantation.

11.1. Induction of Tolerance in Neural Xenografts

There are several lines of evidence suggesting that tolerance can be induced to intracerebral neural xenografts. When embryonic mouse retina (Hankin and Lund, 1987; Lund et al., 1987, 1988; Pollack and Lund, 1990; Subramanian et al., 1995; Young et al., 1989) or neocortex (Marion et al., 1990) is placed in neonatal rat brains, the xenografts can survive for prolonged periods, and form appropriate connections with the host brain. Further evidence suggests

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that the acceptance of neural xenografts by rats might be, at least in part, the result of the induction of tolerance, because full immunocompetence is not developed at the neonatal stage when xenografts are transplanted (Lund et al., 1987). The age of recipient rats seems to play a critical role in the survival of xenografts in this particular model of neural transplantation. When retinal xenografts were transplanted into the brains of neonatal rats older than 8 d, the xenografts were usually rejected (Lund et al., 1987); all the mouse neocortical grafts in the brains were rejected by neonatal rats older than 18 d (Marion et al. 1990).

The use of mAbs against the IL-2 receptor (Honey et al., 1990), or against differentiation molecules on T-lymphocytes (Altera et al., 1991; Chen et al., 1992, 1993; Honey et al., 1991; Pierson et al., 1989; Qin et al., 1990; Wood et al., 1996) has also been demonstrated to induce tolerance to xenografts. Honey et al. (1990) showed that a 10-d treatment of anti-IL-2 receptor led to long-term survival of human fetal dopaminergic (DA-ergic) grafts in rats. Wood et al. (1996) demonstrated that the use of Abs against CD4 and CD8 resulted in an indefinite survival of intracerebral neural xenografts.

11.2. Establishment of Mixed Lymphohematopoietic Chimerism

Studies of bone marrow transplant recipients have shown that tolerance to donor bone marrow, and any other donor tissue, can be induced in the recipient, without completely ablating the recipient's lymphohematopoietic system. The most critical issue for ensuring induction and maintenance of transplantation tolerance is the survival of specific cell types from the donor marrow innoculum in the recipient (Sharabi et al., 1992). This work has been recently extended to xenotransplantation. Sachs and Sablinski (1995) demonstrated that mixed lymphohematopoietic chimerism provides an effective means of inducing long-term specific hyporesponsiveness to concordant xenogeneic skin grafts in rat-to-mouse transplantation (Sharabi et al., 1990). More recently, a similar procedure was extended to the discordant xenograft combination of pig-to-cynomolgous monkey, with encouraging preliminary results (Sachs and Sablinski, 1995). Although no similar study has been reported in neural transplantation, these results suggest that bone marrow chimerism may permit long-term acceptance of discordant neural xenografts in the pig-to-primate combination.

11.3. Thymic Transplantation

Thymic transplantation induces tolerance via a deletional mechanism. Thymic transplantation in pig-to-mouse combinations has been shown to induce a state of tolerance to a discordant xenogeneic thymus donor. Initially, in normal, T- and NK-cell-depleted, and thymectomized mice, renal

subcapsular grafting of fetal pig thymic and liver tissue resulted in efficient mouse thymopoiesis and repopulation of peripheral CD4+ T-cells. Later, in order to examine tolerance to pig Ags in vivo, these mice received skin grafts from the parental line of thymic and liver donor fetuses. Donor-matched pig skin survived permanently in these mice, suggesting that tolerance can be induced across a widely disparate species barrier using thymic transplantation (Lee, 1994). In addition to fetal tissue, neonatal pig thymic and hematopoietic tissue, transplanted to nude mice, can also support the development of functional, specifically tolerant mouse CD4+ T-cells to donor-type pig Ags (Khan et al., 1997). Thymic transplantation thus holds promise as a method of inducing xenogeneic-specific tolerance to neural grafts.

11.4. Inhibition of Co-stimulation

Lack of co-stimulation may also promote tolerance. By inhibiting co-stimulatory molecule expression, or blocking its activity, tolerance may be induced in xenotransplant hosts. Co-stimulatory interactions have been recognized as potential sites of immunosuppression and subsequent prevention of xenograft rejection. Numerous studies involving the inhibition or blockade of co-stimulatory molecules have now been undertaken. The following section will focus on studies in xenotransplantation.

Blocking the CD28–B7 cell co-stimulatory pathway with the fusion protein, CTLA4-Ig, has been extensively used to inhibit alloimmune responses. Promising reports of prolonged survival of allografts with CTLA4-Ig treatment (Balzar et al., 1995; Russell et al., 1996; Sayegh et al., 1995) prompted xenogeneic studies, in which CTLA4-Ig administration was shown to induce long-term, donor-specific tolerance to xenotransplants. Furthermore, significantly prolonged human islet graft survival in mice was induced, in a donor-specific manner, in mice treated with CTLA4-Ig after transplantation (bid, 14 d) (Lenschow et al., 1992). Blockade of the CD28–B7 co-stimulatory interaction inhibits both humoral and cell-mediated immune responses, resulting in increased rat-to-mouse cardiac xenograft survival, and chronic administration of CTLA4-Ig and anti-CD4/CD8 mAbs enhanced this effect (Rehman et al., 1996). Simultaneous blockade of the CD28 and CD40 pathways led to prolonged acceptance of both concordant and discordant xenografts (Elwood et al., 1998); Abs to B7-1 and B7-2 increased the survival of rat cardiac xenografts in mice; inhibited development of antidonor Abs; reduced deposition of C3, IgM, and IgG on xenograft endothelium; and decreased infiltration of activated macrophages, neutrophils, and lymphocytes (Kano et al., 1998). Despite the evidence that CTLA4-Ig can induce tolerance, its mechanism(s) of action in vivo is(are) unknown. However, blockade of CD28–B7 interaction provides a new avenue for prevention of xenorejection.

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Other co-stimulatory interactions have also been targeted. Miwa et al. (1997) showed that rat ICAM-1 and mouse LFA-1 mAb administration, during mouse-to-rat cardiac xenotransplantation, delayed xenoreactive Ab formation and xenograft rejection, and inhibited MLR host serum activation. Administration of a combination of anti-TCR αβ and anti-CD2 mAbs induced specific xenograft tolerance for at least 100 d (Okura et al., 1997). The effects of co-stimulatory molecule blockage in neural xenotransplantation are unknown, but have significant potential.

12. GENETICALLY MODIFIED DONORS

Perhaps the most promising area of research in overcoming the rejection of xenograft tissue lies in the generation of knock-out and transgenic animals as potential tissue donors. In knock-out animals, genes that play a role in xenograft rejection can be deleted through a process of homologous recombination. In transgenic animals, genes can be manipulated to render the donor tissue more human-like in composition. Pigs represent a promising source of genetically modified donor tissue for several reasons: They breed well in captivity, have large litters, and present few ethical objections by the larger community: porcine organs are similar to human organs in both size and efficiency (Kirkman, 1989); furthermore, technology allows us to manipulate the genome of these animals. By genetically modifying donor tissue, long-term xenograft survival may be possible without intensive and possibly toxic immunosuppression. Numerous studies have made great strides in preventing various aspects of xenograft rejection utilizing transgenic porcine tissue.

12.1. Targeting Hyperacute Xenograft Rejection

Although HAR is, for the most part, avoided in neural xenotransplantation, it remains a major obstacle for whole-organ transplants. Transgenic pig technology has been shown to be extremely powerful in preventing the immune mechanisms associated with HAR. In an attempt to mitigate HAR, transgenic animals, expressing human complement regulatory proteins, have been developed. Transgenic pigs expressing proteins such as DAF (White et al., 1995; White, 1996; Cozzi et al., 1997; Storck et al., 1997; Zaidi et al., 1998) and CD59 (Heckl-Ostreicher, 1995; Diamond et al., 1996; Kroshus et al., 1996), have been successful in preventing HAR. Heterotopically transplanted hearts from DAF and CD59 double-transgenic pigs have survived for as long as 69 h in baboons (Byrne et al., 1997). Hearts from DAF transgenic pigs transplanted into monkeys have survived up to 40 d with immunosuppression, exhibiting no signs of rejection when examined histopathologically

(Schmockel et al., 1997). Although these results are promising, long-term survival can only be achieved with immunosuppression at clinically unacceptable doses (Bach, 1997).

Another strategy used to prevent HAR involves blocking the expression of Galα1,3Gal epitopes, which mediate xenoreactive natural Ab binding onto donor tissue. As yet, gene knock-outs have not been achieved in the pig. However, several successful strategies have been used to block expression of this epitope in vitro, and in transgenic mice and pigs. In vitro expression of α1,2-fucosyltransferase (Sandrin et al. 1993) or α2,3-sialyltransferase (Tanemura et al., 1998), which compete with α1,3galactosyltransferase (GALT) for substrate, or N-acetyl glucosaminyl transferase III (Tanemura et al., 1997), which eliminates GALT substrate, markedly reduces Galα1,3Gal surface expression. Furthermore, a transgenic combination of α-galactosidase, which cleaves terminal αGal residues, and α1,2-fucosyltransferase, produced similar Galα1,3Gal reduction (Osman et al., 1997). Teazle et al. (1996) showed a loss of Galα1,3Gal by knocking out the GALT gene in mice.

Transgenic mice expressing α1,2-fucosyltransferase have shown prolonged ex vivo heart survival in a xenograft model (Chen et al., 1998). Additive protection from complement-mediated injury was seen in tissue from a combined GALT knock-out and DAF transgenic mouse (van Denderen et al., 1997). Reduction of the αGal epitope has also been observed in transgenic pigs expressing α1,2-fucosyltransferase (Koike et al., 1997).

A combined GALT knock-out and α1,2-fucosyltransferase transgenic mouse has raised important questions about the possible unmasking of cryptic Ags (Shinkel, 1997). Each of the above transgenic tissues is benefited by decreased Galα1,3Gal expression, which occurs by modifying αGal residues, modifying GALT substrate so that it cannot be processed to form αGal, or processing substrate through other pathways. Each of these methods can result in increased expression of normally suppressed oligosaccharide residues (cryptic Ags) via upregulated alternative pathways. It remains to be determined whether significant natural xeno-Ab recognition of cryptic xeno-Ags will contribute significantly to xenograft rejection in the absence or reduction of Galα1,3Gal, but these interactions will probably be species-specific.

12.2. Targeting Delayed Xenograft Rejection

Various molecular biological approaches are also being used to prevent delayed xenograft rejection. Cell surface molecules, such as thrombomodulin and adenosine triphosphate enzyme (ATPdase), which are lost during EC activation, have been targeted as possible sites of intervention (Bach, 1997, 1998). Attempts to block the upregulation of proinflammatory genes

are also underway. These strategies include the blocking of nuclear factor-κB (NF-κB) by overexpressing the inhibitory factor IκBα (Bach, 1998). More important in neural xenotransplantation is the prevention of xenorejection via MHC molecule recognition. A recent study has demonstrated that the transplantation of VM DA neurons from MHC-I or -II knock-out mice into the striatum of host rats can enhance neuronal survival in comparison to wild-type donors (Duan et al., 1998) beyond 6 wk following transplantation.

12.3. *Cloned Tissue for Neural Xenotransplantation*

Cloned transgenic bovine tissue also represents an important avenue for the future of neural xenotransplantation (Zawada et al., 1998). Cloned transgenic bovine embryos were generated using nuclear transfer from fibroblasts into enucleated oocytes. Implantation of cultured blastocysts resulted in viable gestations beyond 40 d in 42% of the pregnancies. DA-ergic cells expressing β-galactosidase were shown to survive in vitro and produce DA levels similar to wild-type cells. VM tissue from cloned cows expressing the *lacZ* gene was transplanted into rats with unilateral 6-OHDA lesions of the nigrostriatal pathway. Transplanted DA-ergic cells were shown to survive and improve motor performance in immunosuppressed rats. This study demonstrates an exciting new technology that may provide an unlimited supply of identical, genetically altered tissue for transplantation. By cloning donor animals, a uniform cell source is possible, providing thorough characterization and extensive viral screening.

Molecular biology has afforded transplantation a powerful tool to manipulate donor tissue. By expressing a combination of various genes in the donor tissue, long-term xenograft survival may be possible without immunosuppression. Furthermore, recent advances in cloning may allow for a uniform cell source, preventing some of the risks associated with zoonoses.

13. CLINICAL TRIALS

Although many immunological obstacles remain, the first clinical trials of porcine xenotransplantation in PD and HD patients are underway. Fetal porcine VM tissue has been transplanted into the striatum of 12 parkinsonian patients, in an effort to evaluate the safety and ameliorative effects of porcine tissue. In one autopsied patient, graft survival was seen after 7 mo, including the presence of porcine DA-ergic neurons (Deacon et al., 1997). Three of four transplantation sites showed surviving pig cells, and axons from pig neurons showed extensive growth within the graft, as well as extension into the host brain. In assessing the immunological response, only low reactivity of markers for human microglia and T-cells was seen surrounding

the graft. At 12 mo, evaluation of 10 patients showed clinical improvement, with no corresponding adverse effects (Ellias et al., 1998). Porcine cells isolated from the fetal striatal lateral ganglionic eminence have also been transplanted unilaterally into the striatum of 12 patients with HD (Hillaire et al., 1998). These cells were well tolerated, but clinical improvements were not seen during an early posttransplant period. Graft survival in these HD patients is yet to be demonstrated.

Together, these studies represent the first clinical attempts at neural xenotransplantation. Recent advances in understanding of the molecular and cellular mechanisms underlying the immune response to xenotransplantation should facilitate the application of this approach in the treatment of neurodegenerative disorders.

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